

REVERSAL OF ETHANOL-INDUCED HEPATIC STEATOSIS AND LIPID PEROXIDATION BY TAURINE: A STUDY IN RATS

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Abstract — Alcohol (ethanol) was administered chronically to female Sprague–Dawley rats in a nutritionally adequate, totally liquid diet for 28 days. This resulted in significant hepatic steatosis and lipid peroxidation. When taurine was administered for 2 days following alcohol withdrawal it was found to reduce alcohol-induced lipid peroxidation and completely reversed hepatic steatosis. The reversal of hepatic steatosis was demonstrated both biochemically and histologically. Two days following alcohol withdrawal, the apparent activity of the alcohol-inducible form of cytochrome P450 (CYP2E1) was unchanged although total cytochrome P450 content was increased. In addition, alcohol significantly inhibited hepatic methionine synthase activity and increased homocysteine excretion in urine. Although alcohol did not affect the urinary excretion of taurine (a non-invasive marker of liver damage), levels of serum and hepatic taurine were markedly raised in animals given taurine following their treatment with alcohol, compared to animals given taurine alone. There was evidence of slight bile duct injury in animals treated with alcohol and with alcohol followed by taurine, as indicated by raised serum alkaline phosphatase (ALP) and cholesterol. Aspartate aminotransferase (AST) was also slightly raised. The effects of taurine on reversing hepatic steatosis may be due to the enhanced secretion of hepatic triglycerides. It is suggested that increased bile flow as a result of taurine treatment may have contributed to the removal of lipid peroxides. These in-vivo findings demonstrate for the first time that hepatic steatosis and lipid peroxidation, occurring as a result of chronic alcohol consumption, can be reversed by administration of taurine to rats for 2 days.

INTRODUCTION

The liver is considered to be the main organ capable of oxidizing alcohol. Various hepatic enzymes can catalyse the metabolism of alcohol. These pathways are located in different subcellular compartments of the hepatocyte. Thus, the alcohol dehydrogenase pathway (ADH) exists in the cytosol, the microsomal ethanol-oxidizing system (MEOS) is in the endoplasmic reticulum, whereas catalase is located in peroxisomes (Lieber, 1993). Each of these pathways produces specific metabolic and toxic disturbances, and all three result in the production of acetaldehyde, a toxic metabolite.

Chronic alcohol administration leads to proliferation of the smooth endoplasmic reticulum. This ultrastructural alteration is associated with an enhanced activity of the MEOS. MEOS involves a

specific alcohol-inducible form of cytochrome P450 (CYP2E1). CYP2E1 not only catalyses the metabolism of alcohol, but also activates a number of xenobiotics to hepatotoxic and carcinogenic metabolites (Farinati *et al.*, 1989). Alcoholics tend to display tolerance to alcohol as a result of CYP2E1 induction (Lieber, 1997).

Recent reports have indicated that changes in methionine metabolism or methylation in the liver may have an important role in alcohol toxicity. Methionine has to be converted to *S*-adenosylmethionine (SAM) in order to be utilized for functions such as phosphatidylcholine synthesis. SAM also provides a source of cysteine, via the trans-sulphuration pathway, for reduced glutathione (GSH) production, a major hepatoprotective agent against liver injury, including lipid peroxidation. The usefulness of SAM administration in repleting GSH levels has been demonstrated in the baboon (Lieber *et al.*, 1990) and in clinical studies (see Fig. 1) (Vendemiale *et al.*, 1989).

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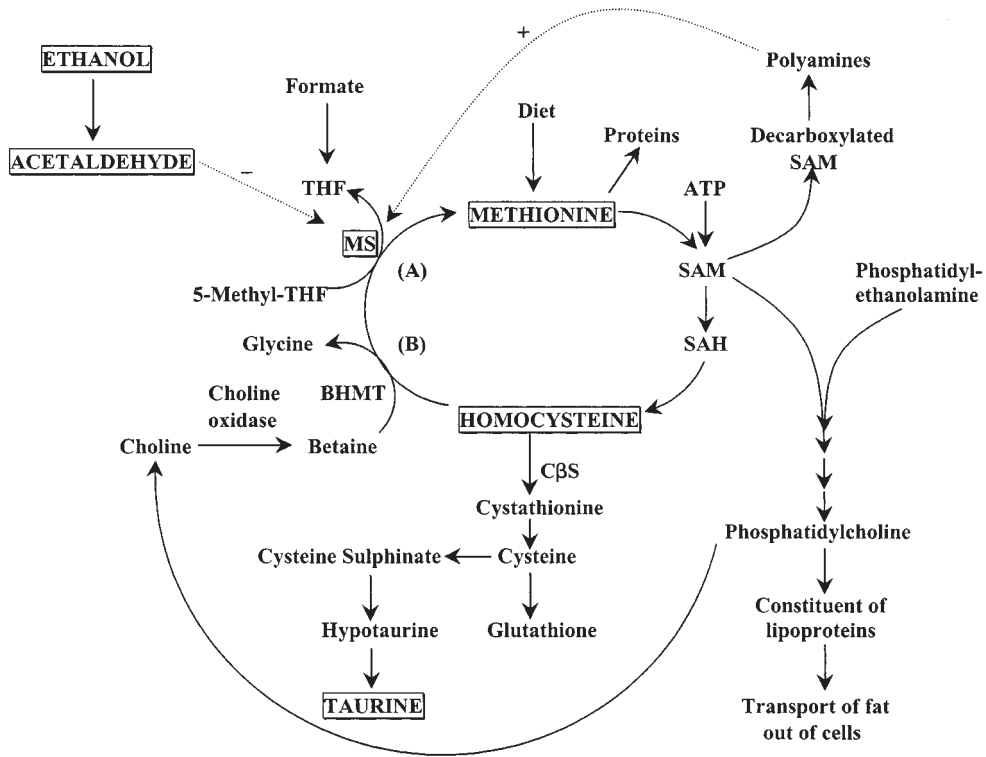


Fig. 1. The methionine cycle and trans-sulphuration pathway.

Enzyme (A) or (B) may operate to convert homocysteine to methionine. Abbreviations: THF, tetrahydrofolate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; MS, methionine synthase; BHMT, betaine homocysteine methyltransferase; C β S, cystathionine β -synthase; +, stimulatory pathway; -, inhibitory pathway. Dotted lines represent modifying reactions.

Methionine may be deficient in alcohol-treated rats, as a result of methionine synthase inhibition (Kerai *et al.*, 1998). Therefore, methionine supplementation has been considered as a treatment for alcoholic liver injury (Finkelstein and Martin, 1986).

Taurine, a sulphur-containing β -amino acid, is the major free intracellular amino acid present in many tissues of man and animals (Huxtable, 1992). Taurine takes part in only a few biochemical reactions of major biological importance in mammalian tissues. These include conjugation with bile acids (Hoffman, 1976), reactions with certain xenobiotics, such as clofibrac acid (Emudianughe *et al.*, 1983) and retinoic acid (Skare *et al.*, 1982). Adult rats can synthesize 80% of their total body taurine and obtain the remainder from the diet (Chesney, 1985). As taurine is synthesized mainly

in the liver from the sulphur-containing amino acids methionine and cysteine, it has not been considered as an essential amino acid (Chesney, 1985). Excess taurine is inert and is excreted into the urine unchanged. Humans have a lower synthetic capacity than rats (also carried out in the liver), although tissue levels are maintained at similar levels to those in the rats through the diet and conservation via the kidneys.

There is significant evidence to suggest that taurine has protective properties both as an endogenous compound and when administered therapeutically (Alvarez and Story, 1983; Nakashima *et al.*, 1983). Recent studies have demonstrated the protective effects of taurine against hepatic steatosis and lipid peroxidation, when co-administered with alcohol for 28 days

(Kerai *et al.*, 1998). The aim of the current study was to show whether the administration of taurine for 2 days following alcohol treatment for 28 days to rats, would reverse the pathological and biochemical lesions induced by alcohol (for example hepatic steatosis and lipid peroxidation). The role of the modulation of CYP2E1 activity and methionine synthase activity in the toxicity was investigated further.

MATERIALS AND METHODS

Chemicals

The following compounds were supplied by Sigma Chemical Company (Poole, Dorset, UK): taurine (synthetic), *o*-phthaldehyde (OPA: HPLC grade), homoserine, (DL)-homocysteine, *S*-adenosyl L-methionine (iodide salt) (SAM), ethanol, sodium dihydrogen phosphate (NaH_2PO_4), Dowex resins, ATP (disodium salt), GSH, firefly lantern extract (luciferase), 5,5'-dithiobis-2-nitrobenzoic acid for measurement of total non-protein sulphhydryls (TNPSH) including GSH. Chromotropic acid, used in the determination of triglycerides, was prepared freshly from 4,5-dihydroxy-2,7-naphthalene disulphuric acid and Zeolite, activated by heating in an oven overnight at 85°C, and these were also obtained from Sigma Chemical Company. Water was of ultra high quality (UHQ), prepared using an Elgastat water system. The AG1-X8 resin (200–400 mesh chloride form) was purchased from BioRad and ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) was obtained from Fluka (Sigma–Aldrich), Gillingham, Dorset, UK. Acetaldehyde was from Aldrich Chemical Company and MTHF (methyltetrahydrofolate, barium salt) (50 $\mu\text{Ci/ml}$) was obtained from Amersham (Little Chalfont, Bucks, UK).

Animals

Female Sprague–Dawley rats (125–150 g) were obtained from Charles River (UK) and acclimatized for 7 days after delivery. Animals were housed in communal cages, fed a rat and mouse maintenance cube diet (691 diet; Quest Nutrition Ltd, Wingham, Kent, UK) and water *ad libitum*. During experiments, animals were housed in individual metabolism cages designed to separate and collect faeces and urine (Techmate Ltd, Milton Keynes, UK), and given powdered diet (691 diet,

Quest Nutrition Ltd, Wingham, Kent, UK) and water *ad libitum* prior to introduction of the liquid diet. Lighting was controlled to give a regular 12 h light–12 h dark cycle (lights on at 08.00); room temperature was maintained at $21 \pm 1^\circ\text{C}$. Urine samples (24 h) were collected over ice and diluted to 25 ml with UHQ water, centrifuged (2000 r.p.m., 10 min, 4°C) to remove hair and food debris and stored (-80°C) in aliquots for later analysis. The liquid diet intake and general condition of the animals were monitored daily and rats were weighed twice a week. Animals were used under the British Home Office regulations.

Liquid diet technique of alcohol administration

Due to the tendency of animals given alcohol to reduce their solid food consumption, animals were given a liquid diet. Chronic alcohol feeding was achieved by incorporation of alcohol in a nutritionally adequate totally liquid diet obtained from Dyets Inc. (Pennsylvania, PA, USA). The liquid diet provided 1 kcal/ml, of which 35% of total calories were derived from fat, 47% from carbohydrates and 18% from protein. Alcohol-treated animals were given diet where maltose–dextrin was isocalorically replaced by alcohol. The alcohol provided 36% of the calories. Animals were started on the diet at a body weight of 125–150 g and alcohol was introduced progressively as 30 g/l of the liquid diet for 2 days, 40 g/l for the subsequent 2 days followed by the final formula containing 50 g/l (Lieber and DeCarli, 1989; Kerai *et al.*, 1998).

Preparation of diet

As vitamin A degrades when mixed with other dry ingredients, vitamins and minerals were incorporated into the liquid diet at the time of preparation using a kitchen-type blender. The diet was kept refrigerated, in the dark and used within 1 week of preparation.

Pair-feeding

The alcohol-fed animals were allowed liquid diet consumption *ad libitum* and their daily intake was monitored. The control animals were then given the same amount of control liquid diet during the following 24 h feeding period. This pair-feeding process was repeated every 24 h. The technique of daily pair-feeding was adopted to assure a strict caloric intake in both alcohol-treated animals

and their individual pair-fed controls (Lieber and DeCarli, 1989).

Study design

Rats ($n = 12$) were treated with alcohol which was administered in the liquid diet for 28 days. Pair-fed control rats ($n = 12$) were also provided with the same liquid diet, but without alcohol. After 28 days, alcohol administration was stopped and alcohol pre-treated animals ($n = 6$) and pair-fed controls ($n = 6$) received the control liquid diet. The remaining alcohol pre-treated animals ($n = 6$) and pair-fed controls ($n = 6$) received the control liquid diet with 3% taurine added. After 2 days of taurine treatment, animals were killed and blood and tissue removed for analysis and microsomes prepared from the liver.

Post-mortem procedure

Animals were exsanguinated from the abdominal aorta under anaesthesia [Hypnorm: Hypnovel: water, 1:1:2, 3.33 ml/kg intraperitoneally (i.p.)] and blood samples were collected into Microtainers® (Becton Dickinson & Co., Rutherford, NJ, USA) for the separation of serum. After standing at room temperature for 45 min, the Microtainers® were centrifuged (13 000 r.p.m., 45 s, MSE minifuge) and stored at -80°C . Serum was analysed for serum enzymes and biochemical parameters using appropriate kits (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany) with a centrifugal IL Monarch 2000 (Instrumentation Laboratory, UK, Ltd). The liver was removed, weighed and ~200 mg taken from the right lobe and immediately homogenized in trichloroacetic acid (TCA, 10% w/v, 4 ml, 4°C), frozen in liquid nitrogen and stored at -80°C for subsequent analysis of ATP. Approximately 200 mg of liver were also taken from the right lobe and immediately homogenized in sulphosalicylic acid (0.2 M, 2 ml, 4°C), frozen in liquid nitrogen and stored at -80°C for subsequent analysis of taurine, total non-protein sulphhydryls (TNPSH) and oxidized glutathione (GSSG).

Biochemical determinations

Taurine. A high performance liquid chromatographic method with fluorimetric detection was used for the determination of taurine in urine, serum and liver tissues, essentially by the method of Waterfield (1994). Taurine was derivatized with *o*-phthaldehyde/2-mercaptoethanol prior to injection

onto a C_{18} column. Isocratic elution of the adduct was carried out using NaH_2PO_4 (0.05 M, pH 5.4) in methanol and water (43:57 v/v). Homoserine was used as an internal standard to facilitate the standardization and quantification of samples. Analysis was completed in 6 min with homoserine and taurine eluting after 3 and 4 min, respectively.

Triglycerides. Hepatic content of triglyceride was determined by a modified method of Butler *et al.* (1962). Briefly, phospholipids were separated from triglycerides by adsorption on a synthetic Zeolite. The triglycerides were then extracted into chloroform, hydrolysed and measured as esterified glycerol with non-esterified samples used as individual blanks.

Lipid peroxidation. Lipid peroxidation, measured as malondialdehyde (MDA) production in liver samples, was determined by the method of Sawicki *et al.* (1963) employing MDA as standard.

ATP. ATP content of liver samples was determined by luciferase-linked bioluminescence in TCA extracts of liver samples using a firefly lantern extract (Jenner and Timbrell, 1994).

Total non-protein sulphhydryls (TNPSH). Liver TNPSH were measured by the method of Ellman (1959) as a measure of liver GSH, which constitutes most (>95%) of the liver TNPSH (DeMaster and Redfern, 1987).

GSSG. Hepatic oxidized glutathione (GSSG) was determined by the method of Griffith (1980) using 2-vinylpyridine to mask GSH.

Microsomal analysis. Microsomes were prepared from livers, essentially as described by Lake (1987). Total cytochrome P450 content of liver samples was determined by the method of Omura and Sato (1964). 4-Nitrophenol hydroxylase (NPOH) activity was determined by the modified method of Prough *et al.* (1978). 4-Nitrophenol is a substrate for the alcohol-inducible CYP2E1. The method relies on the formation of 4-nitrocatechol, which can be detected spectrophotometrically after total ionization under alkaline conditions. The protein content of microsomes was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Homocysteine and cysteine. A high performance liquid chromatographic method with fluorimetric detection was used for determination of total homocysteine and cysteine (oxidized and reduced) in urine and serum, according to Fortin and Genest (1995). Homocysteine and cysteine were reduced

by 10% tri-*n*-butylphosphine in dimethylformamide then derivatized with SBD-F (ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate) at 60°C for 1 h (stable for 1 week at 4°C), prior to injection onto a C₁₈ column. Isocratic elution of the adduct was carried out using sodium acetate (0.1 M), acetic acid (0.1 M) and 2% methanol, pH 4.0. *N*-Acetylcysteine was used as an internal standard to facilitate the standardization and quantification of samples. Analysis was completed in 14 min with cysteine, homocysteine and *N*-acetylcysteine eluting after 2.5, 3.5 and 6 min, respectively.

Methionine synthase. Methionine synthase was measured in the liver cytosol essentially as described by Nicolaou *et al.* (1997). Assay mixtures (total volume 300 µl) contained 50 mM potassium phosphate buffer pH 7.2, 400 µM (DL)-homocysteine, 35 µM SAM, 236 µM MTHF (2658 dpm/nmol), 60 µM hydroxycobalamin, 25 mM DL-dithiothreitol and the enzyme source. Incubations were performed in light-protected stoppered serum vials under nitrogen. Reaction mixtures were pre-incubated for 5 min (at 37°C), prior to the initiation of the reaction by the addition of homocysteine through a syringe. Incubations (at 37°C) were performed for 45 min. The enzyme reaction was terminated by the addition of 400 µl of ice-cold water, and solutions immediately passed through a 0.5×5.0 cm column of Bio-Rad AG1-X8 resin. [¹⁴C]Methionine was eluted with 2 ml of water, collected and quantified by scintillation spectrometry. Protein concentrations were determined with the Bio-Rad protein assay based on the method of Bradford (1976) with bovine serum albumin as standard.

Acetaldehyde. Acetaldehyde in the liver and serum was determined by the method of McCloskey and Mahaney (1981).

Histology

Tissues were fixed in 10.5% (v/v) phosphate-buffered formalin (pH 7.2) and embedded in paraffin wax. Sections (4 µm) were cut and stained with Mayer's haematoxylin and eosin. Frozen liver sections from fixed tissues were cut (10 µm) and stained for lipid with Oil Red O in triethylphosphate with Mayer's haematoxylin as counter stain.

Statistical analysis

Statistical evaluation of data was performed by Duncan's multiple range test to make comparisons

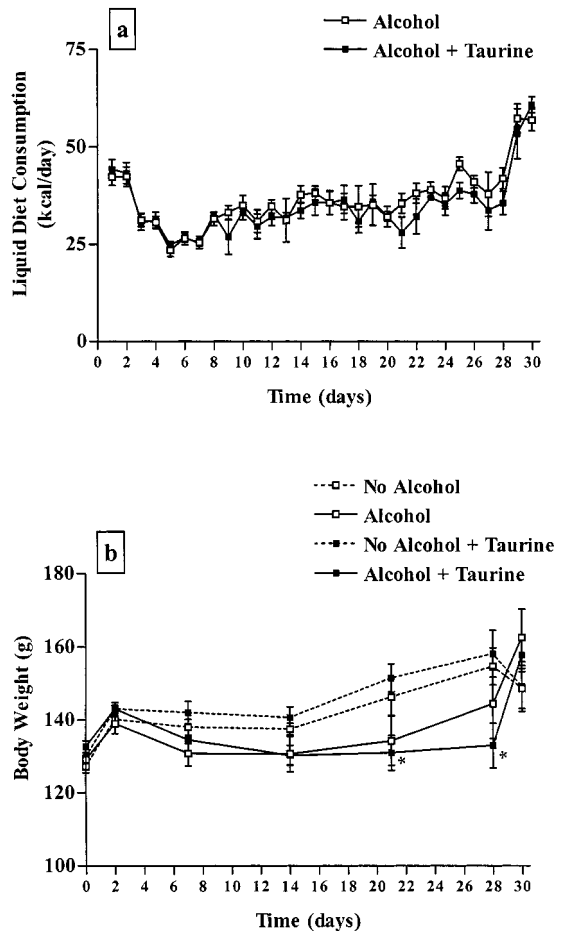


Fig. 2. Liquid diet consumption (a) and body weight of animals (b) during the treatment period.

Results are expressed as means \pm SEM of six animals. Asterisks indicate values significantly different between treated and pair-fed controls (* $P < 0.05$).

between groups. Values quoted are means \pm SEM of six animals. The level of significance was set at ≤ 0.05 .

RESULTS

Liquid diet consumption, alcohol intake and urine volume

There was a gradual decrease in liquid diet consumption as alcohol was progressively increased in

Table 1. The effect of alcohol and taurine treatment on organ weights and serum and hepatic biochemistry

Treatment (days 0–28): Treatment (days 28–30):	no alcohol none	alcohol none	no alcohol + taurine	alcohol + taurine
Total liver weight (g)	4.16 ± 0.24	8.16 ± 0.20***	4.34 ± 0.25	8.09 ± 0.23***
Liver weight (% of body weight)	2.08 ± 0.11	5.05 ± 0.16***	2.91 ± 0.07	5.13 ± 0.07***
Total kidney weight (g)	1.18 ± 0.05	1.38 ± 0.05**	1.21 ± 0.03	1.44 ± 0.05***
Kidney weight (% of body weight)	0.79 ± 0.02	0.85 ± 0.03	0.82 ± 0.03	0.91 ± 0.02*
Serum homocysteine (µmol/l)	5.35 ± 0.26	9.26 ± 1.51**	5.98 ± 0.41	14.28 ± 0.56***c
Serum cysteine (µmol/l)	190 ± 9.14	212 ± 11.57	191 ± 7.54	189 ± 7.49
Liver methionine synthase (nmol/h/mg of protein)	20.0 ± 0.7	13.5 ± 1.2***	16.38 ± 1.1	11.1 ± 1.26**
Serum taurine (mmol/l)	0.37 ± 0.05	0.41 ± 0.04	1.01 ± 0.12 ^d	4.52 ± 0.20*** ^d
Hepatic taurine (µmol/g)	10.7 ± 2.2	5.3 ± 0.8	26.1 ± 3.1 ^d	42.0 ± 3.7*** ^d
Urinary taurine (µmol/kg/24 h)†	38.5 ± 2.9	33.1 ± 3.5	2577 ± 153 ^d	2428 ± 158 ^d
4-NPOH (nmol/min/mg of protein)	0.45 ± 0.10	0.35 ± 0.02	0.42 ± 0.10	0.42 ± 0.05
Total P450 (pmol/mg)	320 ± 24	493 ± 89***	284 ± 63	728 ± 105***c
Hepatic MDA (µmol/g)	1.37 ± 0.21	4.28 ± 0.52***	1.41 ± 0.21	2.94 ± 0.73***c
Hepatic GSH (µmol/g)	4.23 ± 0.77	9.11 ± 0.33***	5.22 ± 0.38	7.10 ± 0.16** ^b
Hepatic GSSG (nmol/g)	62.8 ± 16.0	47.4 ± 3.0	67.2 ± 6.0	43.2 ± 3.5

Measurements were made 30 days after the start of alcohol administration following 2 days of treatment with taurine. Results are means ± SEM of six animals.

Asterisks indicate values significantly different between treated and pair-fed control groups: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Letters 'a', 'b' and 'c' indicate value significantly different between alcohol-treated and alcohol followed by taurine-treated animals: ^a $P < 0.05$, ^b $P < 0.01$ and ^c $P < 0.001$; and ^d $P < 0.001$ for pair-fed groups. All statistical analyses used Duncan's test.

†24 h period before post-mortem.

4-NPOH: hepatic 4-nitrophenol hydroxylase activity.

the liquid diet (Fig. 2a), after which time liquid diet intake remained relatively constant throughout the treatment period. After 28 days, removal of alcohol and introduction of taurine in the liquid diet for 2 days resulted in an increase in liquid diet consumption by all animals. An intake of 12 g/kg (1.6 g alcohol/ rat) in the alcohol-treated animals and 11 g/kg (1.5 g alcohol/ rat) in animals treated with alcohol followed by taurine was achieved. After administration of the liquid diet, the urine volume of all animals was increased (data not shown). The urine volumes were not changed during 28 days of alcohol administration. However, after removal of alcohol and start of taurine treatment, the urine volume was increased in all animals.

Body weights and organ weights

Figure 2b shows the slower body weight gain of alcohol-treated animals compared to pair-fed controls, during the 28 days of liquid diet consumption. After introduction of taurine in the liquid diet for 2 days, the pair-fed controls lost body weight, whereas alcohol-treated animals and

animals treated with alcohol, followed by taurine treatment, gained body weight. There was no significant difference in body weights of animals between groups during the treatment period. Both total and relative liver weights (Table 1) and total and relative kidney weights (Table 1) were significantly raised by alcohol treatment and by alcohol followed by taurine treatment, compared to the pair-fed controls. There was no significant difference in liver weights between alcohol treatment and alcohol followed by taurine treatment.

Serum analysis

Serum alkaline phosphatase (ALP) (a marker of bile duct damage) was significantly raised by alcohol treatment, with a significantly greater increase following taurine treatment, compared with animals treated with alcohol alone (Table 2). However, there was no effect of any of the treatments on serum bile acids (data not shown). Serum cholesterol was also significantly raised by alcohol treatment and by alcohol followed by taurine treatment, as was aspartate aminotransferase (AST) (Table 2). Serum

Table 2. The effect of alcohol and taurine treatment on serum biochemistry

Treatment (days 0–28): Treatment (days 28–30):	no alcohol none	alcohol none	no alcohol + taurine	alcohol + taurine
Cholesterol (mmol/l)	1.48 ± 0.03	2.07 ± 0.18**	1.54 ± 0.09	2.07 ± 0.18*
Albumin (g/l)	31.8 ± 0.64	29.2 ± 0.36***	31.9 ± 0.35	28.9 ± 0.33***
Bilirubin (µmol/l)	2.92 ± 0.22	1.80 ± 0.13*	2.85 ± 0.58	2.00 ± 0.32
Alkaline phosphatase (IU/l)	375 ± 40	926 ± 84***	424 ± 55	1247 ± 137***a
Alanine aminotransferase (IU/l)	38.7 ± 4.7	50.2 ± 1.9	51.0 ± 3.5	61.0 ± 3.5
Aspartate aminotransferase (IU/l)	77.2 ± 5.4	96.5 ± 2.7*	92.4 ± 5.6	133.4 ± 6.2*a
Glutamate dehydrogenase (IU/l)	5.5 ± 1.4	9.5 ± 2.9	7.5 ± 2.1	15.7 ± 2.4

Measurements were made 30 days after the start of alcohol administration following 2 days of treatment with taurine. Results are means ± SEM of six animals.

Asterisks indicate values significantly different between treated and pair-fed control groups: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Letter 'a' indicates value significantly different between alcohol-treated and alcohol followed by taurine-treated animals: ^a $P < 0.05$. All statistical analyses used Duncan's test.

alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH) were slightly increased by treatment with both alcohol and alcohol followed by taurine, although the results were not significant (Table 2). However, alcohol treatment alone significantly reduced total bilirubin, and serum albumin was reduced in both alcohol and in alcohol followed by taurine-treated rats (Table 2).

Urinary, hepatic and serum taurine

There was no effect on urinary taurine excretion during the 28 days of alcohol treatment (Table 1). However, animals consuming the taurine diet for 2 days excreted significantly more urinary taurine, compared to animals on the taurine-free diet, as would be expected. There was no effect of alcohol on hepatic and serum taurine levels (Table 1). In the taurine-treated animals, there were significantly greater levels of hepatic and serum taurine compared to pair-fed controls, as would be expected. However, both hepatic taurine and serum taurine levels were markedly raised in animals treated with alcohol followed by taurine compared to animals treated with taurine alone.

Histological analysis, hepatic and serum triglyceride and lipid peroxidation

Histological examination of liver tissue showed that animals treated with alcohol (Fig. 3c) had developed marked steatosis, compared to pair-fed controls (Fig. 3a). The extent of fat accumulation in animals given alcohol followed by taurine (Fig. 3d)

was distinctly less than in animals treated with alcohol alone, and also less than in taurine-treated animals (Fig. 3b). No method of scoring fat accumulation was used, as triglycerides were also measured biochemically. The hepatocytes in the livers from animals treated with alcohol followed by taurine appeared swollen and vacuolated.

Biochemical measurement of triglycerides showed that alcohol treatment had raised hepatic triglyceride levels significantly above control levels (Fig. 4). However, taurine treatment for 2 days significantly lowered these triglyceride levels to below control values, both for animals which had received alcohol and those which had not. Serum triglyceride levels were significantly raised by alcohol but raised significantly more when alcohol treatment was followed by taurine treatment for 2 days (Fig. 4).

Alcohol treatment also caused a significant increase in lipid peroxidation measured as MDA production, which was significantly decreased in animals treated with taurine following alcohol treatment (Table 1).

Hepatic ATP, GSH, GSSG and microsomal analysis

No difference was detected in the levels of ATP (data not shown) or GSSG between groups after 28 days of alcohol administration (Table 1). However, alcohol and alcohol followed by taurine treatments significantly raised levels of GSH, compared to levels in pair-fed controls, and animals treated

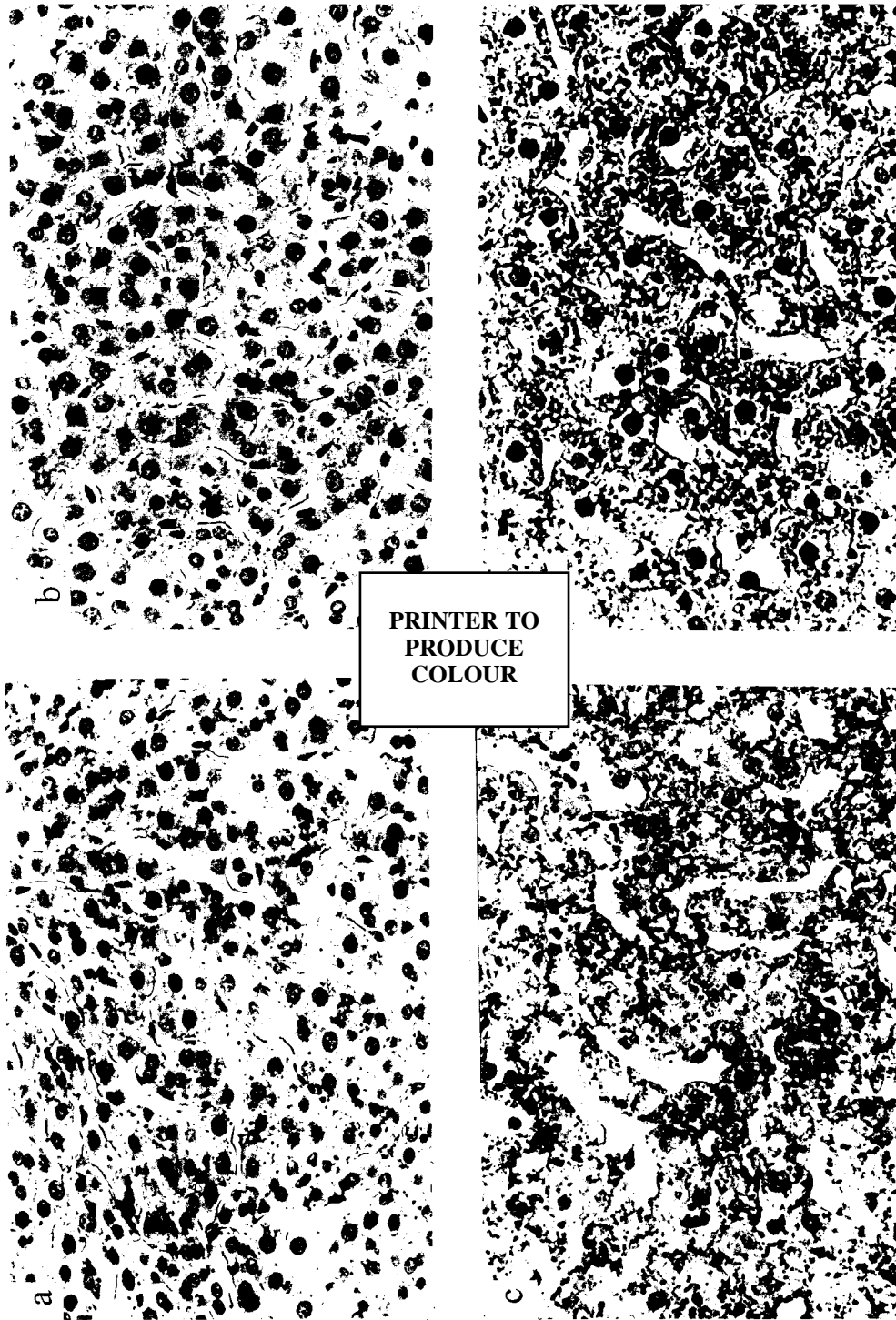


Fig. 3. Photomicrographs of frozen sections of liver ($\times 40$) taken from control rats (a) or rats treated with taurine (b), alcohol (c) or alcohol followed by taurine (d). Frozen sections have been stained with Oil Red O and counterstained with Mayer's haematoxylin. Lipid droplets are stained red.

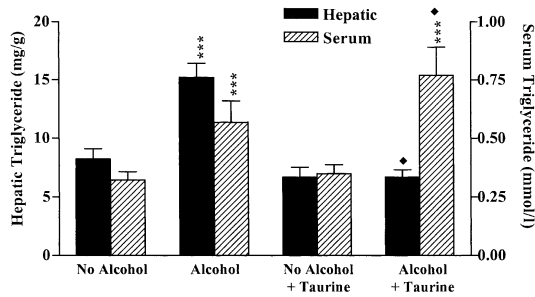


Fig. 4. Hepatic and serum triglycerides in rats at the end of treatment.

Results are expressed as means \pm SEM of six animals. Asterisks indicate values significantly different between treated and pair-fed controls ($***P < 0.001$). Symbols indicate values significantly different between the alcohol-treated and alcohol followed by taurine-treated groups ($\diamond P < 0.001$).

with alcohol followed by taurine had significantly lower levels of GSH compared to animals treated with alcohol alone (Table 1). There was no change in 4-nitrophenol hydroxylase activity 2 days after withdrawing alcohol from the diet in any of the treatment groups (Table 1). Total cytochrome P450 was significantly raised by alcohol treatment and raised significantly more when alcohol was followed by taurine treatment (Table 1).

Serum and urinary homocysteine and cysteine and hepatic methionine synthase

From day 2 of treatment (30 g alcohol/l), urinary homocysteine levels were raised significantly in animals treated with alcohol and alcohol followed by taurine, compared to the pair-fed controls (Fig. 5). The higher levels of urinary homocysteine were maintained in these animals up to day 14 of alcohol treatment. Animals treated with taurine for 2 days had slightly but significantly higher levels of homocysteine and cysteine in the urine. However, alcohol did not affect urinary cysteine levels (data not shown). Although there was no effect of alcohol on levels of serum cysteine, alcohol and alcohol followed by taurine treatment significantly raised levels of serum homocysteine (Table 1). Serum homocysteine levels were significantly higher in animals treated with alcohol followed by taurine than animals treated with alcohol alone. Animals treated with alcohol and alcohol followed by taurine

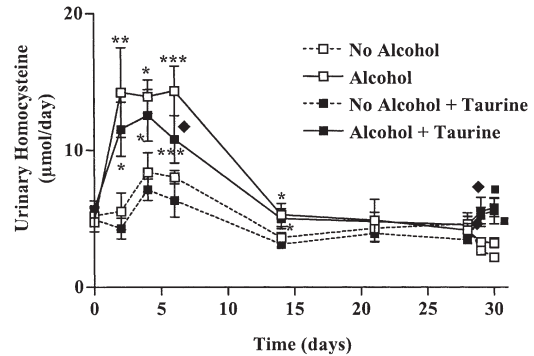


Fig. 5. The effect of alcohol and taurine on levels of urinary homocysteine during the study period.

Asterisks indicate values significantly different between treated and pair-fed controls ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). Symbols indicate values significantly different between the no alcohol-treated and taurine-treated groups and between the alcohol-treated and alcohol followed by taurine-treated groups ($\blacksquare P < 0.01$, $\blacklozenge P < 0.001$).

had hepatic methionine synthase activities which were significantly inhibited, compared to pair-fed controls (Table 1). Taurine-treated animals alone had significantly lower methionine synthase activity compared to the non-alcohol-treated group.

DISCUSSION

The administration of the liquid diet containing alcohol to rats resulted in the accumulation of liver triglycerides and thus provided a model for alcoholic fatty liver as has previously been reported (Vendemiale *et al.*, 1998). We have previously shown that the co-administration of taurine with alcohol to rats alleviates hepatic steatosis and prevents lipid peroxidation (measured as MDA) (Kerai *et al.*, 1998). This study aimed to evaluate whether taurine could reverse alcohol-related hepatic steatosis and lipid peroxidation, once these biochemical changes had developed.

Despite strict isocaloric pair-feeding, alcohol-fed animals did not gain as much weight as their pair-fed controls, although they received diets with the same energy content (Lieber and DeCarli, 1989). This may be due to the oxidation of alcohol without phosphorylation by MEOS. When alcohol

is oxidized to acetaldehyde via the ADH pathway, NADH is generated. However, the oxidation of alcohol via MEOS utilizes NADPH resulting in energy wastage as heat which may explain the slower weight gain of the rats fed the alcohol-containing liquid diets, despite their similar calorific intakes and their increase in weight following alcohol withdrawal. The slower weight gain of the animals in this study compared to those reported by some authors (see, e.g., Lindros and Järveläinen, 1998) could be attributed to strain and/or sex differences of the rats used, or the conditions of housing, such as the room temperature.

The administration of alcohol for 28 days to rats, with or without 2 days of taurine treatment, caused a significant increase in total and relative liver and kidney weights. The increase in the liver weight could not be accounted for by the accumulation of triglycerides as this was <2% of the total increase in liver weight measured in animals treated with alcohol alone. The lack of triglyceride accumulation in animals subsequently treated with taurine also suggests that an additional factor, which is currently unknown, was contributing to the increase in liver weight, although the appearance of the liver suggested that there was oedema of the tissue. The increased total and relative kidney weights suggests an effect of alcohol alone on the kidneys, as previously noted (Kerai *et al.*, 1998).

Alcohol may have caused slight bile duct damage and mild cholestasis, as serum alkaline phosphatase was significantly raised by alcohol although it was raised significantly more when alcohol treatment was followed by taurine. The raised serum cholesterol levels also suggests slight cholestasis (Evans, 1996). The lack of raised serum bilirubin levels suggests that any bile duct injury was slight or that cholestasis was not complete. Hepatic and serum taurine levels were also raised in animals given taurine for 2 days, but the levels were markedly higher in animals given chronic alcohol treatment followed by taurine.

Although taurine is known to increase bile flow (Masuda and Horisaka, 1986), the greater accumulation of liver taurine and serum alkaline phosphatase in alcohol-treated rats post-treated with taurine suggests that taurine may have exacerbated the cholestasis caused by alcohol. The slightly raised level of serum AST, which was greater in animals given taurine following alcohol treatment, may indicate a worsening of the parenchymal

injury. However, the data are inconclusive as ALT and GDH were also moderately raised. However, it is not known what effect alcohol treatment may have had on the sphincter of Oddi which controls the release of bile, although it is thought to reduce the motility of the sphincter (Cullen *et al.*, 1997). An increase in bile volume coupled with a reduction in ability to release bile into the duodenum could increase back pressure and may account for the apparent increase in liver taurine and serum markers of hepatic injury such as alkaline phosphatase. Thus, alcohol treatment caused cholestasis, resulting in bile duct damage (raised alkaline phosphatase), raised serum cholesterol and reduced elimination of liver taurine — effects which may have been enhanced with taurine treatment.

Alcohol treatment increased liver GSH, as previously found (Kawase *et al.*, 1989; Kerai *et al.*, 1998). GSH levels may have been raised as a result of: (a) rebound synthesis of GSH; (b) conversion of homocysteine (which was raised, possibly due to reduced methionine synthase activity) to GSH, or (c) mild cholestasis (Yan *et al.*, 1993). Hepatic levels of GSSG, a marker of oxidative stress, were not significantly changed. There was no apparent effect of alcohol on hepatic levels of ATP.

Hepatic steatosis is the most common pathological change induced by alcohol and is also one of the earliest pathological manifestations of alcoholic liver disease. In the present study, there was a significant accumulation of hepatic triglycerides 2 days following the withdrawal of alcohol. This suggests that levels were probably elevated to a greater extent before the 2-day recovery period. Treatment of animals with taurine for 2 days reversed hepatic steatosis to below control values, as assessed biochemically and histologically. Serum triglycerides were raised by alcohol treatment (as shown previously; Kerai *et al.*, 1998), but this increase was significantly greater in animals treated with alcohol, followed by taurine. This suggests that triglycerides were secreted from the liver (Yan *et al.*, 1993) at an increased rate in animals treated with taurine following alcohol withdrawal. It is likely that this increase in triglyceride efflux was responsible for the reduced hepatic triglyceride levels in animals treated with taurine. The mechanism of this reduction in hepatic triglycerides is currently being investigated *in vitro* in isolated hepatocytes. Thus, taurine not only protects against alcohol-induced hepatic steatosis when

co-administered for 28 days with alcohol (Kerai *et al.*, 1998) but can also reverse hepatic steatosis once it has developed.

Alcohol metabolism is associated with the generation of reactive oxygen species. The polyunsaturated fatty acids that are abundant in cell membranes are susceptible to oxidative damage by free radicals, with consequent lipoperoxide formation, which can lead to the degeneration of membrane phospholipids. The crucial role of lipid peroxidation in the pathogenesis of alcoholic liver injury can be illustrated by the use of antioxidants (Lieber *et al.*, 1994). There is evidence to suggest that taurine may protect against free radical damage (Banks *et al.*, 1991; Huxtable, 1992). Taurine has been shown to suppress lipid peroxidation in the liver of carbon tetrachloride-intoxicated rats (Nakashima *et al.*, 1983) and rabbit spermatozoa (Alvarez and Story, 1983). In the present study, alcohol administration for 28 days caused significant lipid peroxidation (as determined by MDA production) even though measurements were made 2 days following alcohol withdrawal. The levels were reversed by treatment with taurine for 2 days, although the ratio of MDA:liver triglycerides was higher than in the other treatments. There was a slight correlation between individual liver triglyceride and MDA levels ($r^2 = 0.2297$, $P = 0.02$, data not shown). Chronic alcohol treatment of rats raises serum bile acids, which are toxic (Kerai *et al.*, 1998). Taurine, however, is known to increase bile flow (Yan *et al.*, 1993; Seabra and Timbrell, 1997). Thus, an attractive hypothesis to explain the loss of MDA products of lipid peroxidation could be increased rate of removal of bile acids and lipid peroxides by increasing bile flow. There is also evidence that taurine results in the synthesis of phospholipids with a higher proportion of saturated fatty acids and a lower proportion of both polyunsaturated and monounsaturated fatty acids (Yan *et al.*, 1993). Lower levels of unsaturated fatty acids would be expected to result in fewer lipoperoxides being formed, which could have contributed to the lower levels of MDA found in taurine-treated animals.

In our previous study, it was shown that co-administering taurine with alcohol resulted in almost complete inactivation of the alcohol metabolizing cytochrome P450 isoform, CYP2E1 (Kerai *et al.*, 1998). However, in the present study, CYP2E1 activity appeared to be unchanged after

2 days of taurine treatment following alcohol withdrawal, which probably reflects the short half-life of this enzyme (≤ 6 h) (Roberts *et al.*, 1994). Protein measurements will need to be made in future studies to verify these biochemical observations, as 4-nitrophenol hydroxylation is not specific for CYP2E1. However, the total hepatic cytochrome P450 in these animals was actually greater than in animals given alcohol alone, although it is not known which isoenzymes contributed to this increase. There was also no apparent elevation of serum bile acids 2 days following alcohol withdrawal and after taurine treatment, although there was evidence of cholestasis (raised alkaline phosphatase). Thus, by the time cytochrome P450 measurements were made, any inhibition of CYP2E1 by taurine-conjugated bile acids at an earlier time point may have been reversed. As the toxicity of alcohol appeared to be reversed by taurine, without the apparent inhibition of CYP2E1 activity, inactivation of CYP2E1 may not be the main mechanism for the protective effects of taurine seen in the present study.

Homocysteine is a sulphhydryl-containing amino acid that is formed by the demethylation of methionine and is normally metabolized to cysteine, or re-methylated to methionine via methionine synthase (see Fig. 1). Reduced levels of methionine are likely to result in reduced levels of SAM. SAM is used in the methylation of phosphatidyl ethanolamine to phosphatidylcholine which is used in the transportation of triglycerides out of cells. Thus, fatty liver can result from methionine deficiency. The administration of alcohol to rats *in vivo* has been reported to inhibit methionine synthase activity (Barak *et al.*, 1991; Kerai *et al.*, 1998) but not *in vitro* (Sherif *et al.*, 1993). Although *in vitro* studies have failed to show a direct effect of ethanol on methionine synthase activity (0.5–570 mM ethanol), acetaldehyde (in contrast to ethanol or acetate) was found to inhibit methionine synthase activity in a time-dependent manner (Kenyon *et al.*, 1998). This suggests that acetaldehyde, from alcohol metabolism may be responsible for reduced methionine synthase activity *in vivo*. Although acetaldehyde was undetectable in the liver 2 days after alcohol withdrawal in the present study, the inhibition was shown to be irreversible *in vitro* (Kenyon *et al.*, 1998) and therefore, possibly, *in vivo* as well. However, the apparent increase in triglyceride transport from the liver following taurine

administration cannot be attributed to an increase in the activity of this enzyme, as taurine treatment failed to restore methionine synthase activity. It is not known whether taurine can increase the use of betaine as the methyl donor for SAM formation, which could have enabled triglyceride transport to be restored.

The effect of taurine as a protective agent may not be specific. Indeed a recent report (Yin *et al.*, 1998) has shown that glycine (2% in liquid diet) enhances recovery from alcohol-induced liver injury. Although glycine is not a sulphur-containing amino acid, both of these amino acids are part of the methionine/trans-sulphuration pathway. Other sulphur-containing amino acids may also have beneficial effects: methionine through its conversion to *S*-adenosyl-methionine (which may help reverse alcoholic liver damage) and cysteine as a precursor for glutathione, although this is relatively toxic and has poor bioavailability. All of these would ultimately be metabolized to taurine. As far as we are aware, this is the first time that taurine has been shown to reverse alcohol-induced fatty liver and lipid peroxidation when given for 2 days following chronic alcohol administration to rats. The protective effects of taurine may be attributed to enhanced triglyceride secretion from the liver, which may also indirectly have reduced the lipid peroxidation. This is currently under investigation.

Unlike rats, humans have a limited capacity to synthesize taurine and rely more on dietary intake to maintain tissue levels. In view of this, the use of taurine as a dietary supplement, following chronic alcohol consumption, has the potential to be used as a promising therapeutic agent in the treatment of alcoholic liver disease.

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